Neuregulin-1 promotes formation of the murine cardiac conduction system

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Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved June 19, 2002 (received for review May 16, 2002)

The cardiac conduction system is a network of cells responsible for the rhythmic and coordinated excitation of the heart. Components of the murine conduction system, including the peripheral Purkinje fibers, are morphologically indistinguishable from surrounding cardiomyocytes, and a paucity of molecular markers exists to identify these cells. The murine conduction system develops in close association with the endocardium. Using the recently identified CCS-*lacZ* line of reporter mice, in which *lacZ* expression delineates the embryonic and fully mature conduction system, we tested the ability of several endocardial-derived paracrine factors to convert contractile cardiomyocytes into conduction-system cells as measured by ectopic reporter gene expression in the heart. In this report we show that neuregulin-1, a growth and differentiation factor essential for ventricular trabeculation, is sufficient to induce ectopic expression of the *lacZ* conduction marker. This inductive effect of neuregulin-1 was restricted to a window of sensitivity between 8.5 and 10.5 days postcoitum. Using the whole mouse embryo culture system, neuregulin-1 was shown to regulate *lacZ* expression within the embryonic heart, whereas its expression in other tissues remained unaffected. We describe the electrical activation pattern of the 9.5-days postcoitum embryonic mouse heart and show that treatment with neuregulin-1 results in electrophysiological changes in the activation pattern consistent with a recruitment of cells to the conduction system. This study supports the hypothesis that endocardial-derived neuregulins may be the major endogenous ligands responsible for inducing murine embryonic cardiomyocytes to differentiate into cells of the conduction system.

The cardiac conduction system (CCS) is a complex and heterogeneous network of cells within the heart that generates and conducts electrical impulses to enable rhythmic, coordinated contraction of the heart. Lineage-tracing analysis of cardiomyocytes within the looped, tubular chick heart have demonstrated that components of the CCS, including the His bundle, bundle branches, and Purkinje fibers, derive from a cardiomyocyte lineage (1, 2). Within the chick ventricles, sites of cardiomyocyte recruitment to peripheral Purkinje fibers are both subendocardial and periarterial, which led to the hypothesis that a paracrine factor derived from the arterial circulation may be responsible for recruiting "working" myocytes to the CCS (1, 3). It was subsequently shown that endothelin-1 (ET-1), a paracrine factor secreted by endothelial cells in response to shear stress (4), is capable of inducing embryonic chick myocytes to express several CCS markers both *in vitro* and *in vivo* (5, 6).

Although some progress has been made in understanding the molecular signaling pathways regulating avian CCS development, less is known about the regulation of CCS specification in mammalian hearts. In contrast to the avian CCS, the mammalian ventricular CCS is mainly subendocardial, and an association with the arterial system has not been demonstrated. The close proximity of the CCS to the endocardium in both chick and mammalian hearts suggested to us that these specialized endothelial cells may be involved in CCS differentiation in both species. However, no prior study has addressed the ability of endocardial-derived signals to induce CCS differentiation in a mammalian heart.

One obstacle to using a mouse model for addressing this question has been the difficulty in unambiguously identifying CCS components within the murine heart. Cells of the murine CCS are morphologically indistinguishable from the surrounding cardiomyocytes in the tubular heart before 10–11 days postcoitum (dpc). In addition, a paucity of molecular markers for the peripheral Purkinje fibers exists both within the embryonic and fully developed murine heart (7–9). Recently, we identified a line of transgenic mice, (CCS-*lacZ*), in which *lacZ* is expressed within the embryonic CCS beginning between 8.25–8.5 dpc. *LacZ* expression seems to delineate the full extent of the CCS, including the distal Purkinje fiber network, throughout all subsequent stages of development (10). Moreover, by using the highly sensitive technique of optical mapping of electrical activity in embryonic murine hearts, we provided evidence for the functional specialization of components of the CCS as early as 10.5 dpc (10). Therefore, by using the CCS-*lacZ* line of mice, we tested several paracrine factors for their ability to induce CCS differentiation as measured by conversion of cardiomyocytes to *lacZ* positivity. We found that neuregulin-1 (NRG-1) markedly induced ectopic expression of *lacZ* in 8.5- to 10.5-dpc cardiomyocytes. In addition, NRG-1 caused changes in the electrical activation pattern within the heart consistent with this ligand playing a critical role in the recruitment of cells to the CCS.

Materials and Methods

Organ Culture. CCS-*lacZ* mice were maintained on a CD-1 outbred background according to institutional and National Institutes of Health guidelines. CD-1 outbred female mice were mated with CCS-*lacZ* homozygous male mice and the morning of the vaginal plug was designated as 0.5 dpc. Female mice were killed and embryos were dissected from the uterus in ice-cold PBS, followed by removal of the embryonic heart (including atrial, ventricular, and outflow regions). The hearts were cultured in DMEM containing 1% FBS, penicillin and streptomycin (all reagents were from GIBCO/Invitrogen) in 24-well tissue-culture plates, where they continued beating throughout the duration of the experiment. The recombinant peptide containing the β variant of the epidermal growth factor-like domain of NRG-1 was obtained from R & D Systems, and was added to the cultured hearts to reach a final concentration of 2.5×10^{-9} M, except as specified otherwise in the dose–response experiment. Final concentrations of other paracrine factors used were at least 10 times the EC_{50} values, including 10^{-7} M for ET-1, 10^{-5} M for

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: CCS, cardiac conduction system; NRG, neuregulin; BrdUrd, 5-bromodeoxyuridine; dpc, days postcoitum; EGF, epidermal growth factor; ET, endothelin; AV, atrioventricular; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

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 www.pnas.org/cgi/doi/10.1073/pnas.162301699

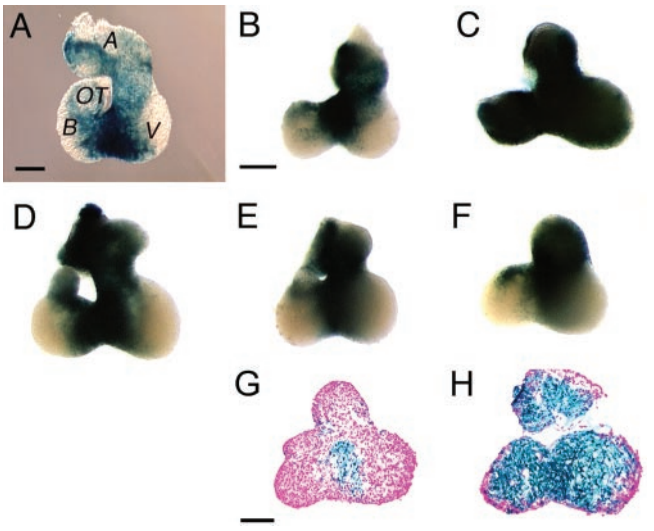

Fig. 1. NRG-1 converts 9.5-dpc embryonic cardiomyocytes to a CCS phenotype. X-Gal staining of a CCS-*lacZ* transgenic heart immediately after removal from a 9.5-dpc embryo (*A*). Highest *lacZ* expression can be seen in the area surrounding the bulboventricular junction in the presumptive ''interventricular ring'', as well as in an area located on the right side of the common atrial chamber which may correspond to the developing sino-atrial node. CCS-*lacZ* hearts were cultured for 48 h in media alone (*B*), or in the presence of paracrine factors including a biologically active NRG-1 peptide (*C*), ET-1 (*D*), angiotensin II (*E*), or insulin-like growth factor-I (*F*), followed by X-Gal staining. *G* and *H* show a representative eosin-stained section from a control and NRG-1-treated heart, respectively. A, common atrium; V, ventricle; B, bulbus cordis; OT, outflow tract. [Bar = 0.2 mm (*A*); = 0.2 mm (*B–F*); = 0.1 mm (*G* and *H*).]

angiotensin II, and 10^{-7} M for insulin-like growth factor-I (all from Sigma-Aldrich), and culture fluid containing these factors as well as NRG-1 was replaced every 12 h in the survey experiment. Control experiments were treated in the same manner, except only vehicle was added. Experiments were terminated by fixation and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining as described (10).

Cell Proliferation Assay. Hearts were harvested from 9.5-dpc CCS-*lacZ* embryos as described above. After 24 h of culture either in the presence or absence of NRG-1, 5-bromodeoxyuridine (BrdUrd; Sigma-Aldrich) was added to the hearts at a final concentration of 30 μg/ml. The hearts were then incubated for an additional 5 h, followed by fixation and X-Gal staining as described (10). Hearts were postfixed for 1 h in 95% ethanol, dropped in 30% sucrose, and embedded in OCT compound (Sakura Finetek, Torrance, CA). Sections were cut at 5 μm, and BrdUrd incorporation was detected by using the 5-Bromo-2′-deoxyuridine Labeling and Detection Kit II (Roche Diagnostics) with FAST Red Substrate (BioGenex Laboratories, San Ramon, CA) for the color detection of alkaline phosphatase. Nuclei were fluorescently counterstained with 4′,6-diamidino-2-phenylindole. Ventricular cells were counted and scored for both BrdUrd incorporation and *lacZ* positivity in a minimum of five sections from several control and NRG-1-treated hearts (3405 total control cells, 5567 total NRG-1 cells).

Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling (TUNEL) Assay. Sections prepared as described above were assessed for apoptosis by using the CardioTACS In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD), followed by fluorescent counterstaining of nuclei with 4′,6-diamidino-2-phenylindole. Ventricular cells were counted and scored for both TUNEL positivity and *lacZ* positivity in a minimum of five sections from several control and NRG-1-treated hearts (4614 total control cells, 4525 total NRG-1 cells).

Whole Embryo Culture. Mouse embryos dissected at 8–8.5 dpc were cultured using the roller system, as described (11, 12). Embryos were cultured, six per 60-ml bottle, at 38°C for 48 h in 5 ml of rat serum to reach the 9.5- to 10-dpc developmental stage. To achieve adequate diffusion of NRG-1 through the visceral yolk sac and pericardial cavity, a higher concentration of NRG-1 peptide (2.5×10^{-8} M) was used.

Optical Mapping of Embryonic Hearts. Hearts were removed from wild-type 9.5-dpc embryos, stained with 1 μM di-4-ANEPPS (Molecular Probes) for 10 min, and subsequently transferred to 37°C Tyrode's solution under an upright microscope equipped with a high-speed CCD camera (Dalsa, Waterloo, Canada). Excitation light from a mercury arc lamp was focused on the hearts while they were beating in sinus rhythm, and the emitted voltage-dependent signal was collected and analyzed as described (10, 13). Images were acquired for 2–4 s at 947 frames per second in the absence of motion-reduction techniques.

Results

Neuregulin-1 Converts Murine Contractile Cardiomyocytes into Conducting Cells. Several paracrine growth and/or differentiation factors known to play a role in embryonic heart development were tested for their ability to convert contractile cardiomyocytes into cells of the CCS. Embryonic hearts from the CCS-*lacZ* line of mice, where expression of the *lacZ* gene delineates the conduction system (10), were excised from 9.5-dpc embryos, and the beating hearts were subsequently cultured in the presence of paracrine factors for 48 h. Conversion of contractile cardiomyocytes to a conduction phenotype was assessed by expression of *lacZ* in ectopic sites within the cultured hearts. A biologically active recombinant peptide containing the β variant of the NRG-1 epidermal growth factor (EGF)-like domain induced ectopic *lacZ* expression in the myocytes of the cultured hearts (Fig. 1 *A–C*). Other paracrine factors, including angiotensin II and insulin-like growth factor-I had little or no effect on *lacZ* expression, whereas ET-1 may have caused a slight induction of ectopic expression (Fig. 1 *D–F*).

Ectopic expression of *lacZ* increased in response to NRG-1 in a dose-dependent manner in the range from 2.5×10^{-11} to 2.5×10^{-9} M (Fig. 2 *A–D*). Exposure to concentrations greater than this did not further increase *lacZ* expression (Fig. 2*E*). Comparison of cultured hearts after 24- and 48-h incubation periods with the NRG-1 recombinant peptide demonstrated a substan-

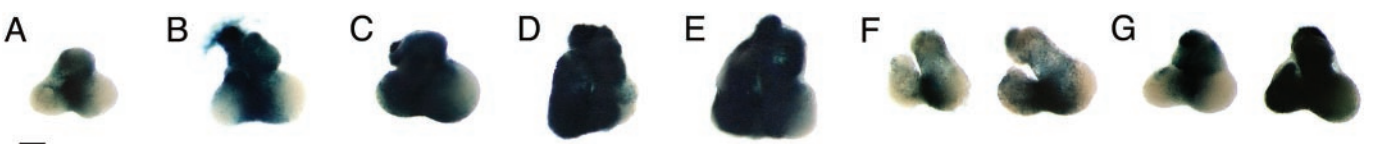

Fig. 2. Dose–response and time course of ectopic *lacZ* expression. CCS-*lacZ* hearts from 9.5-dpc embryos were cultured for 48 h with increasing concentrations of the NRG-1 EGF-like domain: (*A*) none, (*B*) 2.5×10^{-11} M, (*C*) 2.5×10^{-10} M, (D) 2.5×10^{-9} M, or (*E*) 2.5×10^{-8} M, followed by X-Gal staining. CCS-*lacZ* hearts cultured for 24 h (*F*) or 48 h (*G*) in the absence (left heart) or presence of 2.5×10^{-9} M NRG-1 (right heart). [Bar = 0.2 mm (*A–G*).]

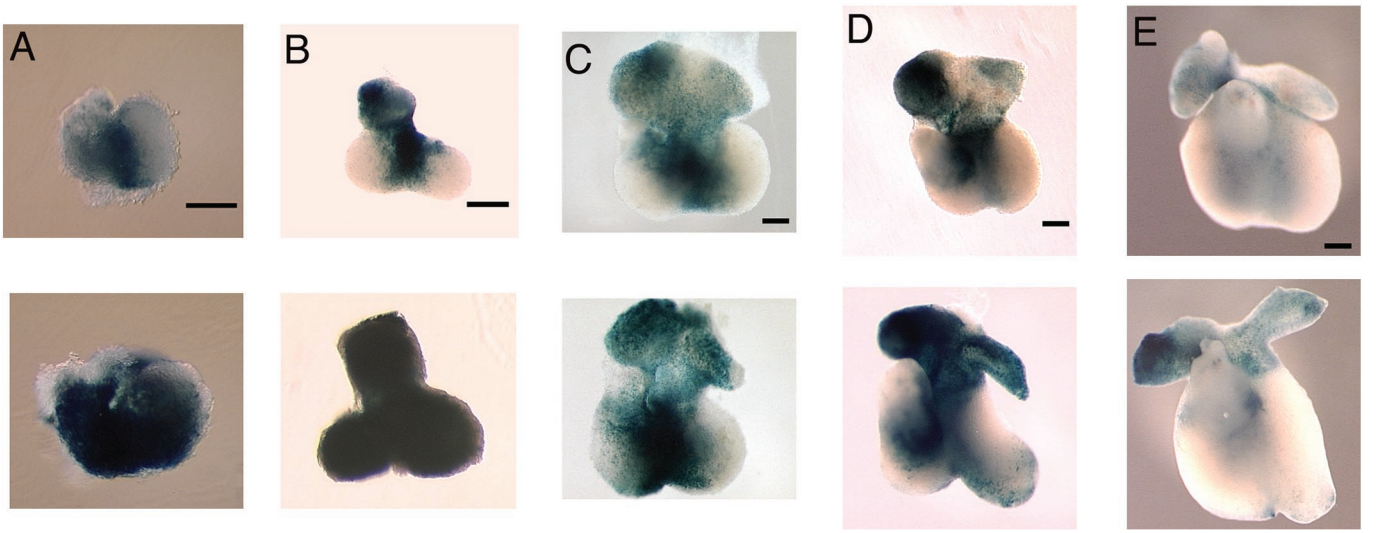

Fig. 3. Response to NRG-1 is developmentally regulated. CCS-*lacZ* hearts from (*A*) 8.5-dpc, (*B*) 9.5-dpc, (*C*) 10.5-dpc, (*D*) 11.5-dpc, and (*E*) 12.5-dpc embryos were cultured for 48 h in the absence (*Upper*) or presence of NRG-1 EGF-like domain (*Lower*) followed by X-Gal staining. Hearts were oriented with the atria located at the top of the picture for each stage except 8.5 dpc, where the atria are not visible. (Bars = 0.2 mm.)

tial effect after treatment for 1 day, whereas after 2 days almost all of the embryonic myocytes expressed the *lacZ* gene (Fig. 2 *F* and *G*). Indeed, compared with control hearts in which 22% of myocytes were *lacZ*+, treatment with NRG-1 for 24 h resulted in 39% *lacZ*+ myocytes, and by 48 h almost all myocytes stained positively (see histologic sections, Fig. 1).

How might NRG-1 treatment so dramatically increase the proportion of *lacZ* positive cells? In addition to its known roles as a cellular differentiation factor, NRG-1 has also been shown to influence cellular proliferation, apoptosis, survival, and fate under certain conditions, depending on the cell type and NRG isoform (14–19). Therefore, we tested whether NRG-1 treatment led to selective proliferation of *lacZ*+ cells. BrdUrd indices were determined in excised hearts cultured in the presence or absence of NRG-1. Despite the marked increase in the proportion of *lacZ*+ cells in NRG-1-treated hearts compared with control hearts, the percentages of *lacZ*+ ventricular cells that were also BrdUrd+ were virtually identical (2.6% in NRG-1-treated vs. 2.8% in control hearts). Thus, selective proliferation of *lacZ*+ cells could not account for the increased proportion of these cells in NRG-1-treated hearts. The proliferation rates for *lacZ*− cells in both control (6.5%) and NRG-1-treated (5.9%) hearts were greater than in *lacZ*+ cells, consistent with a previous report suggesting that myocytes destined to become incorporated into the murine CCS have a slower rate of proliferation than working myocytes (20).

We next examined whether selective loss of *lacZ*− cells through apoptosis occurred in NRG-1-treated hearts, as estimated by TUNEL staining. In control hearts, 14.6% of *lacZ*− cells were TUNEL+. NRG-1 treatment modestly diminished the proportion of *lacZ*− cells that were TUNEL+ to 12.1%. When compared with the *lacZ*− cells, the extent of cell death in *lacZ*+ cells was reduced in both control (4.2%) and NRG-1-treated (2.2%) hearts. Taken together, the cell proliferation and cell death assays suggest that NRG-1 may directly convert embryonic cardiomyocytes to a conduction phenotype.

Response to NRG-1 Is Developmentally Regulated. We have reported that *lacZ* expression is first observed within the hearts of CCS-*lacZ* mice at 8.25–8.5 dpc, and expression continues in cells of the CCS throughout all subsequent stages of development (10). By 13.5 dpc, *lacZ* expression within the CCS seemed similar to at birth, suggesting that patterning of this tissue is largely completed by this stage of development. Therefore, we hypothesized that a paracrine signal(s) responsible for converting myocytes to the CCS should be capable of converting myocytes from younger embryos, but its effects should decline as development proceeds and the CCS becomes fully mature. Consistent with this hypothesis, we found that NRG-1 induced ectopic *lacZ* expression in nearly all myocytes of 8.5- and 9.5-dpc embryonic hearts (Fig. 3 *A* and *B*), but by 10.5 dpc, areas existed within the heart, including the ventricular free walls, that no longer responded to the NRG-1 signal (Fig. 3*C*). In the 11.5-dpc heart, ectopic *lacZ* expression after incubation with NRG-1 was even more restricted, and most myocytes were unaffected (Fig. 3*D*). By this stage, ectopic expression in the ventricle was limited to the apical wall of the future left ventricle. By 12.5 dpc, incubation with NRG-1 resulted in little to no conversion to *lacZ* positivity (Fig. 3*E*). Thus, we conclude that the ability of NRG-1 to convert myocytes to the CCS is restricted to a distinct developmental window.

NRG-1 Induces Ectopic CCS Formation in Treatment of Whole Embryos. To determine whether NRG-1 was capable of inducing ectopic *lacZ* expression within the context of intact, developing embryos, we isolated 8- to 8.5-dpc CCS-*lacZ* embryos and cultured them for 48 h by using techniques described (11, 12), in the presence or absence of NRG-1. NRG-1-treated embryos seemed similar to control embryos on gross examination, and both had reached the 9.5–10.0 dpc developmental stage (Fig. 4*A*). Comparison of *lacZ* expression in control versus NRG-1-treated embryos demonstrated obvious ectopic *lacZ* expression within NRG-1-treated hearts (Fig. 4 *A* and *B*). Other extracardiac tissues that express the *lacZ* reporter gene under the control of regulatory elements contained within the transgene, including the mid/hindbrain and first branchial arch, were unaffected by NRG-1 treatment. In addition, extracardiac tissues known to express ErbB receptors did not up-regulate *lacZ* expression in response to NRG-1 treatment, demonstrating that the transgene is not functioning simply as a NRG-1 reporter gene.

NRG-1 Induces Electrophysiological Changes Within Embryonic Hearts. Our previous study using optical mapping to visualize electrical activation patterns within embryonic hearts suggested that func-

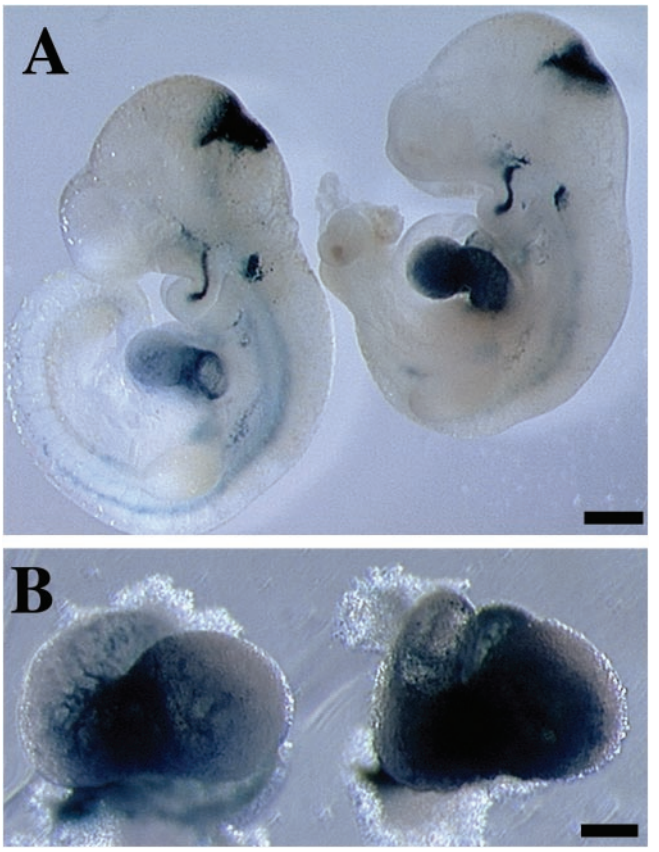

Fig. 4. NRG-1 induces ectopic CCS formation in treatment of whole embryos. CCS-*lacZ* embryos were harvested at 8–8.5 dpc, cultured in the absence (*Left*) or presence (*Right*) of NRG-1, and allowed to develop to 9.5–10.0 dpc. Extracardiac tissues that expressed *lacZ* were unaffected by NRG-1 treatment (*A*), whereas a higher magnification view of the ventricular areas of the hearts clearly shows ectopic lacZ expression within the heart (*B*). [Bar = 0.5 mm (*A*); = 0.2 mm (*B*).]

tional specialization of the CCS already exists by 10.5 dpc, and that the restricted expression of the CCS-*lacZ* transgene at this stage correlates with this electrophysiological specialization (10). To determine whether the broad expansion of *lacZ*+ cells in 9.5-dpc NRG-1-treated hearts is coincident with electrophysiological changes, we used the optical mapping technique to visualize electrical activity in embryonic hearts at this stage. High-resolution imaging of voltage-dependent dye fluorescence was performed on hearts dissected from 9.5-dpc wild-type embryos while the hearts were spontaneously beating in sinus rhythm. After activation of the common atrium and atrioventricular (AV) canal (not shown), the impulse propagated into the primitive ventricle along the dorsal wall ($n = 8$; Fig. 5 *A* and *B*). This location is where both morphological studies and *lacZ* expression in CCS-*lacZ* hearts have identified the first specialized cells of the AV conduction pathway (7, 10). From this initial site of activation along the dorsal ventricular wall, the impulse propagated fastest toward the apex, but also continued to propagate laterally across the entire dorsal surface of the ventricle, followed by the bulbus cordis (Fig. 5*B*). In contrast to the dorsal ventricular surface, initial activation of the ventral surface occurred near the apex and spread fastest toward the AV canal, followed by the remainder of the ventral surface of the ventricle and the bulbus cordis (Fig. 5*A*). Taken together, these activation patterns demonstrate that a preferential conduction pathway exists between the region of the AV canal and ventricle along the dorsal wall.

Next, hearts were cultured for 24 h either in the absence or presence of NRG-1, followed by analysis of the activation patterns. The activation sequence of control cultured hearts was similar to acutely dissected hearts in that the impulse traveled from the AV canal along the dorsal aspect of the ventricle toward the apex, then from the apex along the ventral aspect of the ventricle toward the AV canal ($n = 5$; Fig. 5 *C* and *D*). The heart rate and the delay between earliest atrial and ventricular activation (optically measured pseudoPR interval) were unaffected by neuregulin-1 treatment (not shown). However, the ventricular activation sequence was visibly altered. In 6 of 6 hearts treated with NRG-1 the cardiac impulse not only propagated from the AV canal along the dorsal aspect of the ventricles, but activation from the AV canal region also proceeded along the ventral aspect of the ventricle (Fig. 5 *E* and *F*). In summary, we have provided a description of electrical activity in the 9.5-dpc embryonic mouse heart and demonstrated that NRG-1 treatment results in changes in the pattern of electrical activation consistent with a recruitment of additional cardiomyocytes to a CCS phenotype, as indicated in Fig. 5 *G* and *H*.

Discussion

In this report, we demonstrate that a recombinant peptide encompassing the β variant of the NRG-1 EGF-like domain is capable of inducing a CCS phenotype in embryonic cardiomyocytes. The four identified members of the *neuregulin* gene family encode a diversity of growth and differentiation factors. One common feature shared by all NRGs is the EGF-like domain, which alone is sufficient for receptor binding and bioactivity (15, 21–23). Two of the four NRG family members, NRG-1 and NRG-2, are expressed by endocardial cells in the embryonic heart; however, each has been shown to stimulate distinct signaling pathways (24, 25). NRG-1 is found mainly in the ventricles, whereas NRG-2 is expressed more highly in the atria (24, 26).

The *neuregulin-1* gene undergoes extensive alternative splicing, encoding more than 15 distinct isoforms, some of which are membrane-bound and some are soluble. In addition, soluble forms of NRG can also be generated by proteolytic processing of transmembrane precursors (27, 28). Of the three major NRG-1 isoforms, the Type I isoforms are the only isoforms expressed before embryonic day 10, when induction of the murine ventricular CCS is likely to initiate (29). By 8.5 dpc, expression of NRG-1 Type I can be seen within the ventricular endocardial cells, where it continues to be expressed throughout embryonic development (29). Therefore, the expression pattern of NRG-1 Type I is both spatially and temporally consistent with a potential role for this ligand in CCS induction.

The receptors for NRG-1 expressed by embryonic cardiomyocytes are ErbB2 and ErbB4, which are members of the EGF receptor family of tyrosine kinases. This signaling pathway is essential for proper embryonic heart development. NRG-1, ErbB2, and ErbB4 homozygous mutant mice all die between 10 and 11 dpc, apparently because of the absence of ventricular trabeculae, the region within the heart where the CCS develops (26, 30–32).

We show in this report that treatment of embryonic 9.5-dpc CCS-*lacZ* hearts with the NRG-1 EGF-like domain for 24 h results in substantial conversion of cardiomyocytes to *lacZ* positivity, but 48 h was required for maximal conversion (Fig. 2 *F* and *G*). Similarly, NRGs produced by motor neurons at the neuromuscular junction initiate a signaling cascade in muscle that induces acetylcholine receptor synthesis. The timeframe for substantial acetylcholine receptor gene induction was shown in chick primary myotubes to be at least 12–24 h, with continuous phosphorylation of ErbB receptors required throughout this timeframe (33).

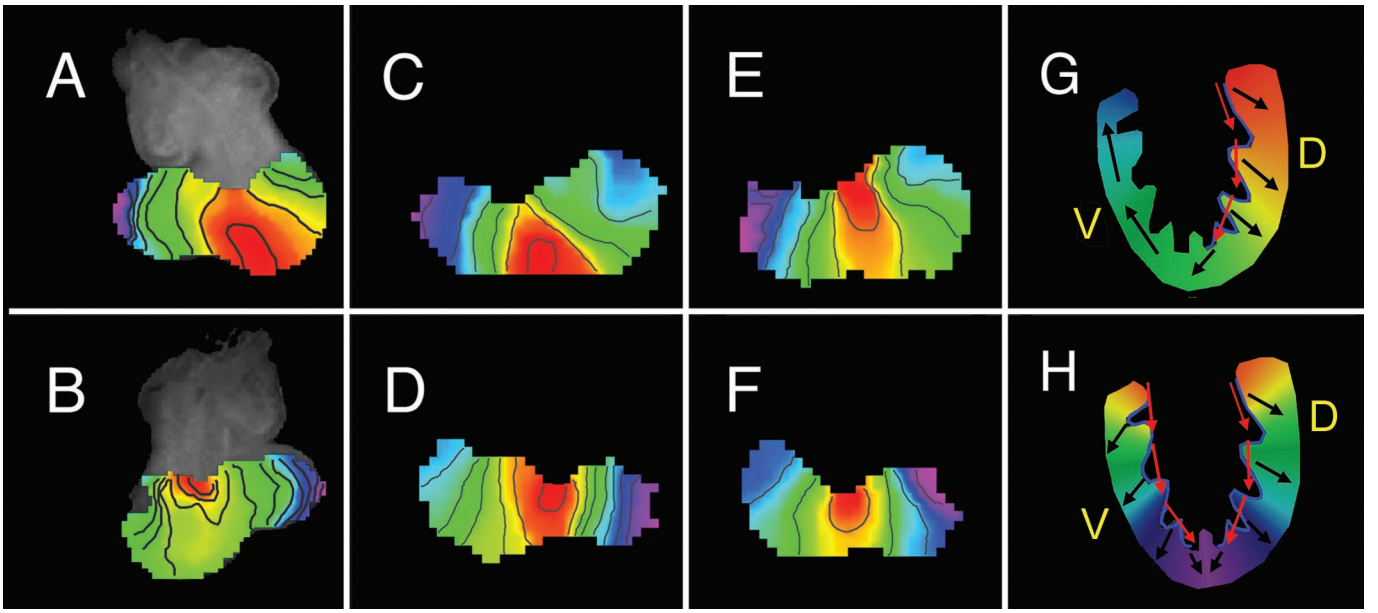

Fig. 5. NRG-1 induces electrophysiological changes within embryonic hearts. Activation maps of 9.5-dpc wild-type hearts from either the ventral (*A*, *C*, *E*) or dorsal (*B*, *D*, *F*) surfaces are shown. Acutely dissociated hearts (*A*, *B*); hearts cultured for 24 h in the absence (*C*, *D*) or presence (*E*, *F*) of NRG-1. Activation progresses from red to purple, with isochrone lines drawn every 1 ms. Model depicting CCS-dependent activation of the ventricles in control (*G*) and NRG-1 (*H*) treated hearts. A sagittal view is shown with ventral (V) and dorsal (D) surfaces indicated. The CCS is indicated by the blue region along the subendocardial surface; activation sequence is indicated by colors progressing from red to purple. Red arrows indicate activation of the CCS; black arrows indicate activation of the working myocardium.

Similar to the effects of ET-1 observed on avian conduction system differentiation, we observed a distinct temporal window of sensitivity to NRG-1 treatment, with a developmental decline in the conversion of embryonic cardiomyocytes to *lacZ* positivity. By 10.5 dpc, a significant number of cardiomyocytes within the cultured hearts were no longer responsive to NRG-1, and by 12.5 dpc almost all were unresponsive (Fig. 3). This decline in responsiveness may be essential for preventing cardiomyocytes from being recruited to the CCS at late developmental stages when the CCS is already organized and functioning.

One interesting question that remains unanswered is why only a subset of the 8.5- and 9.5-dpc myocardium differentiates into CCS *in vivo*, given the seemingly pan-endocardial expression of NRG-1 (29). We hypothesize that a differential sensitivity of myocytes to the inductive effects of NRG-1 exists, with myocytes near the interventricular region being the most sensitive and those near the free walls, the least sensitive. Supporting this hypothesis is our finding that when hearts are bathed in varying doses of NRG-1 (see Fig. 2), the interventricular myocardium is most sensitive and induction is progressively weaker toward the free walls (Fig. 2 *A–E*). In addition, shorter incubation times are required for CCS induction in the interventricular region compared with the free walls (Fig. 2 *F* and *G*). A possible explanation for this difference in sensitivity within the 9.5-dpc heart could be a gradient in the expression of ErbB receptors or of a component of the downstream signaling pathway within the myocardium. Similarly, progressively restricted expression of either the ErbB receptors or downstream signaling factors throughout development may play a role in the developmental decline in response to NRG-1 discussed above.

This report is a description of the ventricular activation pattern in the 9.5-dpc murine heart and demonstrates that, already at this early developmental stage, preferential conduction proceeds from the AV canal into the ventricle along the dorsal wall. These observations are consistent with earlier morphological studies that have determined that the first specialized cells of the AV conduction pathway are seen along the dorsal wall as early as 9 dpc (7). They also agree with our studies of the CCS-*lacZ* mice, which visualize this same region as early as 8.5 dpc (10). In agreement with the near simultaneous contraction of the ventricle seen in the rat heart at this stage (34), we see rapid activation of the mouse ventricular region and demonstrate that impulses propagate most rapidly in the region of the interventricular ring, a location where *lacZ*+ cells delineate the developing His-Purkinje system. By 10.5 dpc the ventricular activation pattern is markedly altered, with earliest activation of the ventricle occurring near the apex, rather than from the AV canal along the dorsal wall. These data suggest that functional insulation of the proximal ventricular CCS from the working myocardium takes place between 9.5 and 10.5 dpc (35).

We used semiquantitative reverse transcription–PCR to examine whether alterations might occur in gene expression consistent with expansion of the CCS after NRG-1 treatment of 9.5-dpc hearts for 48 h (data not shown). However, given the paucity of CCS markers in the murine heart before 13.5 dpc, this approach was not particularly revealing. We anticipate that genetic profiling of NRG-1-treated versus control hearts will identify more useful early genetic markers of CCS specialization.

We generated an additional 16 transient transgenic lines by using the same construct as in the CCS-*lacZ* line, but none had expression of the reporter gene in the CCS (data not shown). This result supports our initial suggestion that CCS-*lacZ* expression is under the transcriptional control of the locus of integration, rather than *Engrailed-2* regulatory elements included within the transgene proper. We have recently prepared a genomic library from the CCS-*lacZ* mice and cloned the site of integration. No obvious candidate genes exist that might account for the uniquely restricted pattern of β-galactosidase activity in the CCS-*lacZ* mice. Nonetheless, we will continue our efforts to determine the mechanisms accounting for this intriguing pattern of expression in the developing and mature CCS.

In summary, we have extended our studies on functional maturation of the murine CCS to include visualization of ventricular activation patterns as early as 9.5 dpc. Moreover, we have identified a genetic pathway that implicates NRG-1 as a key ligand promoting formation of the murine CCS. Future studies

will be required to define the precise genetic pathway through which NRG-1 acts and to identify those endogenous genes that account for CCS functional specialization.

We thank Dr. John T. Fallon, Otis Defreitas, and Yolanda Ortega of the Cardiovascular Histopathology Core at Mount Sinai School of Medicine for their help on this project. This work was supported by Grants HL64757 (to G.I.F.) and HL58442 (to S.A.R.) from the National Institutes of Health, a Scientist Development Award from the American Heart Association (to G.E.M.), and the Donaghue Medical Research Foundation (to S.A.R.). G.I.F. is a recipient of the Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

1. Gourdie, R. G., Mima, T., Thompson, R. P. & Mikawa, T. (1995) *Development (Cambridge, U.K.)* **121,** 1423–1431.
2. Cheng, G., Litchenberg, W. H., Cole, G. J., Mikawa, T., Thompson, R. P. & Gourdie, R. G. (1999) *Development (Cambridge, U.K.)* **126,** 5041–5049.
3. Mikawa, T. & Fischman, D. A. (1996) *Annu. Rev. Physiol.* **58,** 509–521.
4. Yoshizumi, M., Kurihara, H., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T. & Yazaki, Y. (1989) *Biochem. Biophys. Res. Commun.* **161,** 859–864.
5. Gourdie, R. G., Wei, Y., Kim, D., Klatt, S. C. & Mikawa, T. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6815–6818.
6. Takebayashi-Suzuki, K., Yanagisawa, M., Gourdie, R. G., Kanzawa, N. & Mikawa, T. (2000) *Development (Cambridge, U.K.)* **127,** 3523–3532.
7. Viragh, S. & Challice, C. E. (1977) *Dev. Biol.* **56,** 382–396.
8. Viragh, S. & Challice, C. E. (1977) *Dev. Biol.* **56,** 397–411.
9. Viragh, S. & Challice, C. E. (1980) *Dev. Biol.* **80,** 28–45.
10. Rentschler, S., Vaidya, D. M., Tamaddon, H., Degenhardt, K., Sassoon, D., Morley, G. E., Jalife, J. & Fishman, G. I. (2001) *Development (Cambridge, U.K.)* **128,** 1785–1792.
11. Sturm, K. & Tam, P. P. (1993) *Methods Enzymol.* **225,** 164–190.
12. Zhao, Z. & Rivkees, S. A. (2001) *Dev. Dyn.* **221,** 194–200.
13. Vaidya, D., Tamaddon, H. S., Lo, C. W., Taffet, S. M., Delmar, M., Morley, G. E. & Jalife, J. (2001) *Circ. Res.* **88,** 1196–1202.
14. Bacus, S. S., Huberman, E., Chin, D., Kiguchi, K., Simpson, S., Lippman, M. & Lupu, R. (1992) *Cell Growth Differ.* **3,** 401–411.
15. Carraway, K. L., 3rd, Soltoff, S. P., Diamonti, A. J. & Cantley, L. C. (1995) *J. Biol. Chem.* **270,** 7111–7116.
16. Daly, J. M., Jannot, C. B., Beerli, R. R., Graus-Porta, D., Maurer, F. G. & Hynes, N. E. (1997) *Cancer Res.* **57,** 3804–3811.
17. Syroid, D. E., Maycox, P. R., Burrola, P. G., Liu, N., Wen, D., Lee, K. F., Lemke, G. & Kilpatrick, T. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9229–9234.
18. Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P. & Anderson, D. J. (1994) *Cell* **77,** 349–360.
19. Zhao, Y. Y., Sawyer, D. R., Baliga, R. R., Opel, D. J., Han, X., Marchionni, M. A. & Kelly, R. A. (1998) *J. Biol. Chem.* **273,** 10261–10269.
20. Thompson, R. P., Germroth, P. G., Gourdie, R. G., Thomas, P. C., Barton, P. J. R., Mikawa, T. & Anderson, R. H. (1995) in *Developmental Mechanisms of Heart Disease*, eds. Clark, E. B., Markwald, R. R. & Takao, A. (Futura Publishing, Armonk, NY), pp. 269–279.
21. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., *et al.* (1992) *Science* **256,** 1205–1210.
22. Carraway, K. L., 3rd, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C. & Cerione, R. A. (1994) *J. Biol. Chem.* **269,** 14303–14306.
23. Kita, Y., Mayer, J., Zamborelli, T., Hara, S., Rohde, M., Watson, E., Koski, R., Ratzkin, B. & Nicolson, M. (1995) *Biochem. Biophys. Res. Commun*. **210,** 441–451.
24. Carraway, K. L., 3rd, Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M. & Lai, C. (1997) *Nature (London)* **387,** 512–516.
25. Sweeney, C., Fambrough, D., Huard, C., Diamonti, A. J., Lander, E. S., Cantley, L. C. & Carraway, K. L., 3rd (2001) *J. Biol. Chem.* **276,** 22685–22698.
26. Meyer, D. & Birchmeier, C. (1995) *Nature (London)* **378,** 386–390.
27. Lemke, G. (1996) *Mol. Cell. Neurosci.* **7,** 247–262.
28. Burden, S. & Yarden, Y. (1997) *Neuron* **18,** 847–855.
29. Meyer, D., Yamaai, T., Garratt, A., Riethmacher-Sonnenberg, E., Kane, D., Theill, L. E. & Birchmeier, C. (1997) *Development (Cambridge, U.K.)* **124,** 3575–3586.
30. Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. & Lemke, G. (1995) *Nature (London)* **378,** 390–394.
31. Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. & Hauser, C. (1995) *Nature (London)* **378,** 394–398.
32. Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E. & Theill, L. E. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4833–4838.
33. Li, Q. & Loeb, J. A. (2001) *J. Biol. Chem.* **276,** 38068–38075.
34. Lloyd, T. R. & Baldwin, H. S. (1990) *Pediatr. Res.* **28,** 425–428.
35. James, T. N. (2001) *Prog. Cardiovasc. Dis.* **43,** 495–535.